Protein-binding properties of the putative AP-1 and ATF sequences in the feline immunodeficiency virus long terminal repeat

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Feline immunodeficiency virus (FIV) is a member of the genus Lentivirus, family Retroviridae (Miyazawa & Mikami, 1993). Gene expression in lentiviruses is modulated by cellular factors through transcriptional regulatory sequences in the long terminal repeat (LTR) and by virus-encoded regulatory proteins (Cullen & Greene, 1989). In the U3 region of the FIV LTR, many putative binding sites for enhancer proteins (Cullen & Greene, 1989). In the U3 region of the FIV LTR and by virus-encoded regulatory factors through transcriptional regulatory sequences in the 1993). Gene expression in lentiviruses is modulated by cellular reported (Phillips et al., 1997). For gel-supershift assays demonstrated that the AP-1 and ATF sites had similar protein-binding properties. The effects of internal deletions of AP-1 and/or ATF sites on the basal promoter activity were also examined. Although deletion of either site moderately reduced activity, a mutant deleted in both sites had dramatically reduced activity. Therefore, we suggest that these two sites co-operatively regulate transcriptional activity of the promoter.

Electrophoresis-mobility-shift assays with nuclear extracts from a feline renal cell line and a T-lymphoblastoid cell line revealed that the AP-1 and ATF sites of feline immunodeficiency virus (FIV) TM2 strain had similar protein-binding properties to those of FIV Petaluma strain and consensus sequences of AP-1 and ATF sites, and that nuclear factors binding to these sites differed between the two cell lines. Cross-competition and gel-supershift assays demonstrated that the AP-1 and ATF sites had similar protein-binding properties. The effects of internal deletions of AP-1 and/or ATF sites on the basal promoter activity were also examined. Although deletion of either site moderately reduced activity, a mutant deleted in both sites had dramatically reduced activity. Therefore, we suggest that these two sites co-operatively regulate transcriptional activity of the promoter.

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The ATF site is critical for the cAMP-induced response mediated by protein kinase A (Sparger et al., 1992). A DNase I footprinting assay using nuclear protein extracts from a feline T-lymphoma cell line revealed specific nuclear protein-binding to the putative AP-1, ATF and C/EBP sites (Thompson et al., 1994). Previously, we and others demonstrated genetic diversity in the LTR among FIV isolates (Thompson et al., 1994; Yamada et al., 1995). Although cross-competition between putative AP-1 and ATF motifs of FIV UK8 strain was suggested (Thompson et al., 1994), it is still not known whether there are differences in the proteins that bind to the putative AP-1 or ATF site among different FIV isolates or different cell types. Moreover, it remains unclear whether the putative AP-1 or ATF sites are really bound by AP-1- or ATF-like proteins.

In the present report, we examined the specific nuclear proteins that bind to the AP-1 and ATF sites using nuclear extracts from a feline renal cell line [Crandell feline kidney (CRFK) cells] and a feline T-lymphoblastoid cell line (MYA-1 cells). Furthermore, nuclear proteins binding to the AP-1 or ATF site were characterized using anti-c-Jun/AP-1 and anti-ATF-1 antibodies.

CRFK cells (Crandell et al., 1973) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated FCS and antibiotics. MYA-1 cells (Miyazawa et al., 1989) were maintained in RPMI 1640 medium supplemented with 10% FCS and antibiotics. Infectious molecular clones of FIV Petaluma and TM2 strains were used in this study (Olmedo et al., 1989; Maki et al., 1992). Double-stranded oligonucleotides used as probes were:TM2/AP-1 (5’ ATATGAGTCAGGTTAATGCT 3’), TM2/ATF (5’ TGGCCGATGACGTATCTTGC 3’), F14/AP-1 (5’ GCATGACTCATAGTTAAAGCGC 3’), F14/ATF (5’ TGGCTAATGACGTATAAATGCT 3’), Cons/AP-1 (5’ CGCTTGTGAGTGACCGGGA 3’), Cons/ATF (5’ AGAGATTGCTGGTUCAGTGAGTAGACGTAAGCTGTTCC 3’). The positions of the AP-1, ATF and Sp-1 sites are underlined. Electrophoresis-mobility-shift assays (EMSA) were performed as described previously by Ikeda et al. (1997). For gel-supershift
assays, we used rabbit anti-human c-Jun polyclonal IgG and mouse anti-human ATF-1 monoclonal IgG as anti-AP-1 and anti-ATF antibodies, respectively (Santa Cruz Biotechnology). Gel-supershift assays were performed as described by Ikeda et al. (1997).

To determine whether the putative AP-1 and ATF sites really function in factor recognition, EMSA were performed using nuclear extracts from CRFK and MYA-1 cells. The double-stranded oligonucleotide containing the AP-1 or ATF sequence of FIV TM2 strain (TM2/AP-1 or TM2/ATF, respectively) was labelled with $^{32}$P and incubated with the nuclear extracts. When TM2/AP-1 was used, two and three sizes of binding complexes were observed in CRFK (A and B) and MYA-1 (F, G and H) cells, respectively (Fig. 1a). When TM2/ATF was used, three sizes of binding complexes were observed in CRFK (C, D and E) and MYA-1 (I, J and K) cells (Fig. 1a). Complexes B, E, F and K could be detected only after long exposure. When homologous unlabelled competitors were added to the reaction mixtures, complexes A, B, C, D, F, G, H, I and J were competed. However, when a heterologous oligonucleotide containing the consensus Sp-1 binding site (Cons/Sp-1) was used, these complexes were not affected. These results indicated that nuclear factors specifically binding to the putative AP-1 or ATF sites were present in CRFK and MYA-1 cells and that the binding factors were similar but not the same between the two cell lines. In addition, complex E was affected equally by unlabelled homologous and heterologous competitors while complex K was not affected even by
addition of unlabelled competitor, suggesting that formation of complexes E and K was due to non-specific DNA–protein interactions.

To compare the protein-binding properties of TM2/AP-1 or TM2/ATF with those of the consensus AP-1 or ATF sequence (Cons/AP-1 or Cons/ATF, respectively) and the putative AP-1 or ATF site of FIV Petaluma strain (F14/AP-1 or F14/ATF, respectively), the six oligonucleotides were labelled and incubated with nuclear extracts from CRFK and MYA-1 cells. Although slight differences were observed, the binding patterns of the three AP-1 or ATF sequences were similar to each other (data not shown), suggesting that similar protein-binding properties operate on the putative AP-1 or ATF sequences of the FIV strains and the consensus sequences.

For further characterization of the binding properties, competition assays were performed using unlabelled TM2/AP-1, TM2/ATF, F14/AP-1, F14/ATF and Cons/Sp-1 as competitors. Competition analyses clearly demonstrated that protein binding to labelled TM2/AP-1 was inhibited by addition of cold TM2/AP-1, TM2/ATF, F14/AP-1 and F14/ATF but not Cons/Sp-1 in CRFK cells (Fig. 1b). However, binding to labelled TM2/ATF was reduced only by addition of cold TM2/ATF and F14/ATF in CRFK cells (Fig. 1b). Similar results were also obtained in competition assays of labelled TM2/AP-1 or TM2/ATF performed with MYA-1 cell extracts (Fig. 1b). Furthermore, although competition assays using labelled Cons/AP-1 or Cons/ATF in CRFK extracts also showed similar results, complex formation of Cons/AP-1 with CRFK nuclear extracts was not completely inhibited by addition of cold TM2/AP-1 or F14/AP-1 probe (Fig. 1c). It appeared that the binding of nuclear proteins to Cons/AP-1 is more efficient than to the binding sequences of FIV isolates. Additionally, when we performed the competition assays on Cons/AP-1 and Cons/ATF with MYA-1 cell extracts, the results were similar to those observed with CRFK cell extracts (data not shown).

To characterize the nuclear factors binding to the AP-1 or ATF sites, gel-supershift assays using anti-AP-1 and anti-ATF antibodies were performed with CRFK extracts. When labelled TM2/AP-1 was used as a probe, supershifted bands were observed upon treatment with anti-ATF as well as anti-AP-1 antibodies (Fig. 2a, lanes 2 and 3). Similar results were obtained when labelled Cons/AP-1 was used as a probe (Fig. 2a, lanes 5 and 6). When labelled TM2/ATF was used as a probe, a supershifted band was demonstrated with anti-ATF antibody (Fig. 2a, lane 9). Although it was faint, a supershifted band was also observed with anti-AP-1 antibody (Fig. 2a, lane 8). When labelled Cons/ATF was used as a probe, similar supershifted patterns were observed after treatment with the antibodies (Fig. 2a, lanes 11 and 12). No supershifted band was revealed by these antibodies when the putative C/EBP sequence of FIV TM2 strain was used as a probe (data not shown), suggesting that nuclear proteins were specifically recognized by these antibodies. Furthermore, when supershift experiments were
Bam dramatically reduced the LTR-directed CAT activity (Fig. 3). Of either the AP-1 or ATF site reduced the LTR-directed CAT examined in CRFK cells by the CAT assay. Although deletion specific mutations of the AP-1 and ATF sites for promoter activity, the effects of each of the site-specific deletions of the AP-1 and ATF sequences were recognized by both AP-1- and ATF-like proteins.

pTM1 CAT, which contains the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of the FIV LTR, was described by Kawaguchi et al. (1991) and pHd CAT, used as a negative control plasmid, was described by Shibata et al. (1990). Internal-deletion mutants of the FIV LTR, pAP-1 CAT and pATF CAT, were described by Kawaguchi et al. (1995). pAP-1dATF CAT was constructed by exchanging a BamHI–NheI fragment of pAP-1 CAT with a BamHI–NheI fragment of pATF CAT. For transfection of plasmid DNA into CRFK cells, the cells were plated in six-well plates. Seven µg of plasmid DNA was transfected by the calcium phosphate co-precipitation method (Graham & van der Eb, 1973). Forty-eight hours after transfection, cell monolayers were harvested for the CAT assay. Cell extracts were prepared as described previously by Gorman et al. (1982). CAT activity was measured by the solvent partition method as described by Neumann et al. (1987) and is presented as the net d.p.m. of products formed per hour.

To determine the significance of the putative AP-1 and ATF sites for promoter activity, the effects of each of the site-specific mutations of the AP-1 and/or ATF site(s) were examined in CRFK cells by the CAT assay. Although deletion of either the AP-1 or ATF site reduced the LTR-directed CAT activity at most two- to threefold, deletion of both sites dramatically reduced the LTR-directed CAT activity (Fig. 3).

FIV can infect various cell types in vivo, such as T lymphocytes, monocytes/macrophages, brain cells and other cells (Dow et al., 1990; Beebe et al., 1994). Basal promoter activity of the FIV LTR was reported to be dependent on cell type (Miyazawa et al., 1992; Sparger et al., 1992). In the present study, we demonstrated the different protein-binding properties of the putative AP-1 and ATF sequences of FIV between CRFK and MYA-1 cells by EMSA (Fig. 1) as we previously reported with the C/EBP sequence of the FIV TM2 strain (Kawaguchi et al., 1995). From these results, the different promoter activities of the FIV LTR in various cell lines might be due to the different properties of enhancer proteins which bind to the LTR in these cells. It is highly probable that the FIV LTR participates in controlling virus replication at the intracellular level.

As shown in Fig. 1, proteins that bind to the labelled AP-1 sequences were affected by addition of cold AP-1 or ATF oligonucleotides. On the other hand, the proteins that bind to the ATF sequences were unaffected. These results might be because the ATF sequence has a stronger affinity for proteins which bind to both AP-1 and ATF sites. As shown in Fig. 2, the putative AP-1 and ATF sequences of FIV were bound by both AP-1- and ATF-like proteins. These data might reflect the similarity between the core recognition sequences [TGA(C/G)TCA for AP-1 and TGACGTCA for ATF] and cross-family dimerization of Fos/Jun and ATF/CREB (Hai & Curran, 1991). Analyses of site-specific mutants have shown that the putative AP-1 site is required for activation by a phorbol ester or c-Fos, and that the putative ATF site is the target for cAMP-induced responses (Sparger et al., 1992; Miyazawa et al., 1993). However, low positive responses remained even when an FIV LTR lacking the AP-1 site was treated with phorbol ester or c-Fos and when an LTR lacking the ATF site was treated with an inducer of cAMP (forskolin) (Sparger et al., 1992; Miyazawa et al., 1993). Furthermore, a positive response to phorbol ester decreased even when the ATF site was deleted, and the effect of treatment with forskolin was reduced even when the AP-1 site was deleted (Sparger et al., 1992). Additionally, the replication rate and the cytopathogenic activity of the AP-1 binding site-deleted mutant virus were almost the same as those of the wild-type virus in feline T-lymphoblastoid cells (Miyazawa et al., 1993). Here we demonstrated that mutations of both sites showed drastic reduction of the basal promoter activity although deletion of either of the sites had less effect on the activity. From these observations, it was suggested that the two sites could play...
complementary roles in certain cells. Recently, we reported that the AP-1 binding site-deleted mutant virus grows less efficiently than wild-type virus in vivo (Inoshima et al., 1996). Further analysis in vivo using infectious molecular clones lacking the ATF site or both AP-1 and ATF sites will be necessary to assess the roles of the AP-1 and ATF sites in virus replication.

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References


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